REMARKS

Claim 4 has been amended based on the disclosure at, e.g., Examples such as Example 4 (see the third line of Example 4). Claim 8 has been amended to correct a typographical error. Claims 9 and 10 have been canceled. Claim 11 has been amended in view of the cancellation of claim 10 and the amendment of claim 4. Claim 12 has been amended in view of the amendment of claim 4.

Entry of the above amendment is respectfully requested.

Obviousness Rejections

Claims 4-8 and 12 are rejected under 35 U.S.C. 103(a) as being unpatentable over Shimomura et al. (previously cited) in view of Maruyama et al. (previously cited), Zhao et al. (Journal of Applied Polymer Science 2003 90:1846-1850) and Berge et al. (Physical Review A 1990 41:6893-6902). Claims 4-8 and 12 are rejected under 35 U.S.C. 103(a) as being unpatentable over Shimomura et al. in view of Maruyama et al., Nishikawa et al. (previously cited), and Berge et al. Claims 9-11 are rejected under 35 U.S.C. 103(a) as being unpatentable over Shimomura et al. in view of Maruyama et al., Nishikawa et al., and Berge et al. as applied to claims 4-8 and 12 above, and further in view of Kazakov et al. (US PGPub No. 2003/0044455). Claims 9-11 are rejected under 35 U.S.C. 103(a) as being unpatentable over Shimomura et al. in view of Maruyama et al., Zhao et al., and Berge et al. as applied to claims 4-8 and 12 above, and further in view of Kazakov et al.

In response, Applicants note initially that the Examiner argues that it would have been obvious to one of ordinary skill in the art to exchange DPPC for the amphiphilic polymer in the

invention of Shimomura et al. as the simple substitution of one known element for another to yield a predictable result (see the first paragraph on page 5 and the paragraph bridging pages 6-7 in the Office Action). A similar argument can be found at the bottom of page 7 of the Office Action. In addition, the Examiner indicates that Maruyama et al. highlight that the amphiphilic compound can also be non-polymeric (see page 6 of the Office Action).

However, Applicants submit that this is not correct.

If the Examiner were correct, a honeycomb film could be prepared by using any phospholipid instead of the amphiphilic polymer in Shimomura, since phospholipids are amphiphilic in any event.

Actually, though, this is not the case. See Biomaterials, 27, 2006, 1797-1802, a copy of which is attached hereto. As shown in Table 1, among various phospholipid molecules, only dioleoylphosphatidylethanolamine (DOPE) could form a honeycomb structure under the given condition.

The reason for this may be in the fact that DOPE has sufficiently high interfacial tension. As the authors assert, interfacial tension is largely dependent on the chemical structure of the phospholipids, and therefore it is an unpredictable feature. See the abstract of Soft Matter, 2009, 5, 2037-2041, a copy of which is attached hereto. Without knowing such a feature of DOPE and the technical relationship between the formation of a honeycomb structure and the strength of interfacial tension, which is still only but a hypothesis, an ordinary artisan would not have selected DOPE from among other low molecular weight compounds including phospholipids.

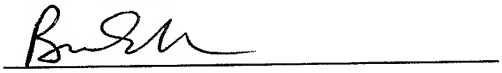
Therefore, Applicants submit that the invention as recited in the amended claims is not obvious over prior art, and withdrawal of these rejections is respectfully requested.

Conclusion

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



Bruce E. Kramer
Registration No. 33,725

SUGHRUE MION, PLLC
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

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Biodegradable honeycomb-patterned film composed of poly(lactic acid) and dioleoylphosphatidylethanolamine

Yukako Fukuhira[a,*], Eiichi Kitazono[a], Takami Hayashi[a], Hiroaki Kaneko[a], Masaru Tanaka[b], Masatsugu Shimomura[c], Yoshihiko Sumi[a]

[a]*Department of Tissue Engineering Development, Innovation Research Institute, Teijin Ltd., 4-3-2, Asahigaoka, Hino, Tokyo 191-8512, Japan*
[b]*Creative Research Initiative Sousei, Hokkaido University, N21W10, Sapporo 001-0021, Japan*
[c]*Nanotechnology Research Center, Research Institute for Electronic Science, Hokkaido University, N21W10, Sapporo, 001-0021, Japan*

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Abstract

Honeycomb-patterned films have been reported to be useful for scaffolds of cell culture in tissue engineering. In the present study, we investigated a new compound, dioleoylphosphatidylethanolamine (DOPE), a naturally derived phospholipid having unsaturated fatty acid moieties, as a surfactant for fabricating honeycomb-patterned poly(D,L-lactide) (PLA) film. Only DOPE among commercially available phospholipids was useful as a surfactant, and it showed good solubility in PLA/chloroform solution and an excellent property for fabricating honeycomb-patterned film (the concentration of DOPE was from 0.2% to 20% by weight based on the weight of PLA). The pore size of the honeycomb was uniform, and all pores were interconnected with each other. The contact angle of water on the honeycomb-patterned film was affected by the amount of DOPE. Time-of-flight secondary ion mass spectrometer (TOF-SIMS) data suggested that DOPE was concentrated on the surface of the honeycomb-patterned film. To investigate cell proliferation and adhesion on the honeycomb-patterned film, NIH3T3 fibroblast cells were cultured on the film. The NIH3T3 cells adhered well on the honeycomb-patterned PLA film with DOPE (PLA–DOPE) and showed good cell proliferation compared to that on honeycomb-patterned PLA film fabricated with a copolymer (CAP) of dodecylacrylamide and ω-carboxyhexylacrylamide (PLA–CAP).

These results suggest that the honeycomb-patterned PLA–DOPE can be applicable as a scaffold for cells with better profiles in comparison with PLA–CAP.

Keywords: Phospholipid; Honeycomb-patterned film; Polylactic acid; Cell adhesion; Surface modification; Tissue engineering

1. Introduction

In recent years, the micro-size structure has been widely applied to electronic materials [1], catalysts [2], the separator for blood cells [3,4], and cell culture substrates [5–7], etc. These are known to be prepared by lithography [8], soft lithography [9], phase separation of block copolymers [10], and so on. In regard to the application of the micro-size structure to the separator for blood cells or cell culture substrates, it is recognized that the surface structures of materials have considerable influence on the adhesion, migration and proliferation of cells. Since the micro-size structure on the material surface is important to control cell behavior, much attention has been directed toward technology for fabricating the material surface.

Some authors have reported that the honeycomb-patterned micro-size structure on a polymer surface can control cell activity arbitrarily. This honeycomb pattern was prepared by a simple casting method [11–13]. More particularly, the polymer solution was cast on a petri dish, water droplets self-assembled on the polymer solution by adding humid air, and the honeycomb pattern was observed on polymer film after drying. Cell proliferation and adhesion on the honeycomb pattern were influenced by the pore size of the honeycomb structure.

It is possible to produce a honeycomb structure on biodegradable polyester films made from poly(lactic acid)

*Corresponding author. Tel.: +81 42 586 8327; fax: +81 42 587 5511.
E-mail address: y.fukuhira@teijin.co.jp (Y. Fukuhira).

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or poly(ε-caprolactone) [3]. A honeycomb-patterned film on biodegradable polyester is useful as a cell culture substrate. The complex of cultured cells and honeycomb-patterned biodegradable polymer film is expected to be useful for tissue engineering. To form the honeycomb pattern on polymer film, a surfactant is essential to stabilize the water droplets and acts as a template of the honeycomb pattern on the surface of the polymer solution. The surfactant must have amphiphilicity, because it contributes to the stability of the water droplet at the polymer solution–water interface by forming a micelle-like structure.

Polymers having this property, such as the amphiphilic polymers and polyion complexes, can form the honeycomb structure by themselves [5–7,11]. Nevertheless, an amphiphilic poly (acrylamide) copolymer, CAP, which is derived from dodecylacrylamide and ω-carboxyhexylacrylamide, has been reported as the only surfactant useful for fabricating the honeycomb pattern on biodegradable polyester so far [3]. It is a synthetic polymer, and the metabolic pathway in the body has not been made clear. If a honeycomb-patterned film composed of biodegradable polyesters can be fabricated using a naturally derived or biodegradable surfactant, it can be used preferably as a scaffold for tissue engineering or prosthesis in the human body. However, that kind of surfactant for fabricating a honeycomb pattern on biodegradable polyesters has not been reported at all.

The purpose of this study was to investigate an optimal biodegradable surfactant for fabricating the honeycomb pattern on biodegradable polyester, Poly(D,L-lactic acid) (PLA) film. In this study, we focused on naturally derived phospholipids such as phosphatidylcholines (PC) and phosphatidylethanolamines (PE), and examined them as surfactants to fabricate the honeycomb structure on PLA film. The cell proliferation and adhesion on the PLA film was observed by using NIH3T3 cells.

2. Materials and methods

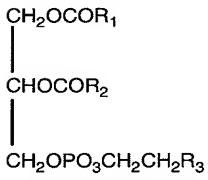
2.1. Fabrication of the honeycomb-patterned film

PLA (Mw = 200,000 [14], Lacty #9031, Shimadzu, Kyoto, Japan) was dissolved in chloroform (Wako Pure Chemical Industries Ltd., Osaka, Japan) at a concentration of 5 mg/mL at room temperature. Dioleoylphosphatidylethanolamine (DOPE) (COATSOME ME-8181, NOF Co., Tokyo, Japan) was added in various concentrations from 0.1% to 100% by weight based on the weight of PLA. Similarly, other phospholipids (DLPC, DMPC, DPPC, DSPC, DOPC, DMPE, DPPE, and DSPE) shown in Table 1, were added to the polymer solution by wt% concentration (5, 10 and 20 wt%). The honeycomb-patterned films were fabricated by casting the polymer solution under blowing with air (about 80% humidity, 1–8/min.) at room temperature in air of 30% humidity. The casting volume of the polymer solution was 5 mL/petri dish (10 cm in diameter). The honeycomb-patterned films for the cell culture were prepared on cover glasses (φ: 15 mm, Matsunami Glass Industrial Ltd., Tokyo, Japan), which were placed in the petri dishes, and produced the same pore size (3 μm). The pore size of the honeycomb-patterned film can be controlled by the temperature, humidity, gas flow rate, casting volume, and casting concentration [15]. The honeycomb-pattern was observed by an optical microscope (BX51, Olympus Co., Tokyo, Japan) and FE-SEM (S-5200, Hitachi High Technologies, Tokyo, Japan). The contact angles of the honeycomb-patterned films were measured (CA-S, Kyowa Interface Science, Saitama, Japan). The residual DOPE in the honeycomb-patterned film after ethanol washing was measured by ^{1}H-NMR (A-600, JEOL, Tokyo, Japan). The surface chemical composition of the honeycomb-patterned film was observed by Time-of-flight secondary ion mass spectrometer (TOF-SIMS IV, ION-TOF GmbH, Germany).

Table 1
The capability of phospholipids to form the honeycomb-patterned structure on PLA film

	Phospholipids	5 wt%[a]	10 wt%	20 wt%
DLPC	(R_1, $R_2 = C_{11}H_{22}$, $R_3 = N^+(CH_3)_3$)	Phase separation	Phase separation	Phase separation
DMPC	(R_1, $R_2 = C_{13}H_{26}$, $R_3 = N^+(CH_3)_3$)	Phase separation	Phase separation	Phase separation
DPPC	(R_1, $R_2 = C_{15}H_{30}$, $R_3 = N^+(CH_3)_3$)	Phase separation	Phase separation	Phase separation
DSPC	(R_1, $R_2 = C_{17}H_{34}$, $R_3 = N^+(CH_3)_3$)	Phase separation	Phase separation	Phase separation
DOPC	(R_1, $R_2 = C_{17}H_{32}$, $R_3 = N^+(CH_3)_3$)	Opaque	Opaque	Opaque
DMPE	(R_1, $R_2 = C_{13}H_{26}$, $R_3 = NH_3$)	n.d.[b]	n.d.	n.d.
DPPE	(R_1, $R_2 = C_{15}H_{30}$, $R_3 = NH_3$)	n.d.	n.d.	n.d.
DSPE	(R_1, $R_2 = C_{17}H_{34}$, $R_3 = NH_3$)	n.d.	n.d.	n.d.
DOPE	(R_1, $R_2 = C_{17}H_{32}$, $R_3 = NH_3$)	Honeycomb	Honeycomb	Honeycomb

[a]Surfactant concentration by weight based on the weight of PLA.
[b]n.d. = not dissolved in chloroform.



Phospholipid

2.2. Assessment of cell proliferation and adhesion

NIH3T3 cells (American Type Culture Collection, VA, USA) were used for assessing cell proliferation and adhesion on the honeycomb-patterned films. For the cell culture, sterilization of the honeycomb-patterned films was done by 70% ethanol washing. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Grand Island, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, UT, USA), penicillin (100 U/mL) and streptomycin (50 mg/mL). The cells were seeded on the honeycomb-patterned films coated on cover glasses (113 mm^2) at a density of 3.2×10^2 cells/cm^2 in a 12-well culture plate and cultured in a humidified environment of 5% CO_2 at 37 °C. After 24 and 48 h culture periods, the cell proliferations were measured by alamarBlue™ (Alamar Biosciences Inc., CA, USA) assay [16]. The alamarBlue™ was added to the wells in an amount equal to 10% of the total culture medium volume, and then the well was incubated for 2 h. Aliquots of 100 μL/sample were measured by a fluorescence plate reader (Wallac 1420 ARVOsx, PerkinElmer, Inc., MA, USA) set at excitation and emission wavelengths of 530 and 590 nm, respectively.

For assessing the cell adhesion on the honeycomb-patterned films, NIH3T3 cells were grown for 14 days and the media were changed every 2 days. Cell adhesions were observed by SEM (JSM-5310, JEOL, Tokyo, Japan) and FE-SEM, and the coverage of cells on the honeycomb-patterned film was calculated by image analysis software (nexusNewQube, Sumisho Electronics Co., Ltd., Tokyo, Japan).

2.3. Statistics

The data were expressed as means and standard deviations (SD). Comparisons of means between groups were performed by using the Student's unpaired t-test, and a p value of less than 0.05 was considered significant.

3. Results and discussion

Honeycomb-patterned films were prepared by a casting method from the polymer solution (5 mg/mL) under blowing with highly humid air at room temperature, and a phospholipid was used as the surfactant. Table 1 shows the capability of phospholipids to form the honeycomb-patterned structure on PLA film. PEs having saturated fatty acid moieties, such as DMPE, DPPE and DSPE, could not be dissolved in the polymer solution. DOPE (a PE having unsaturated fatty acid moieties), DOPC (a phosphatidylcholine having unsaturated fatty acid moieties) and all PC having saturated fatty acid moieties such as DLPC, DMPC, DPPC and DSPC were dissolved in the polymer solution. The solubility of each surfactant was checked visually. In this study, we used only soluble phospholipids for the honeycomb-patterned film fabrication. Film with PC having saturated fatty acid moieties, DLPC, DMPC, DPPC and DSPC, which were not observed to form the honeycomb-patterned structure on the surface, showed only phase separation. Film with phosphatidylcholine having unsaturated fatty acid moieties, DOPC, also did not form the honeycomb structure and only showed an opaque surface. DOPE was the only case in which PLA film showed the honeycomb-patterned structure on the surface. These phenomena were observed at surfactant concentrations of from 5% to 20% by weight based on the weight of PLA, as shown in Table 1.

The mechanism of the honeycomb-patterned formation has been described as follows: After placing a droplet of the polymer solution on the substrate, chloroform starts to evaporate. This leads to the cooling of the polymer solution and enables micron-size water droplets to condense onto the polymer solution. By evaporating the chloroform solution, the water droplets get close to being hexagonally packed. After evaporating the chloroform solution, the water droplets evaporate and the honeycomb-patterned structure is observed.

The balance between the alkyl chain length and size of the hydrophilic group of surfactants, as well as the solubility of the surfactants in the polymer solution, are important in fabricating the honeycomb-patterned film. DOPE has unsaturated alkyl chains and the volume of the hydrophilic group is comparatively smaller than that of PC. It has the advantage of forming reversed micelle structures in the water and oil phase [17]. DOPE as a surfactant contributes to the stability of the water droplets by self-assembly to the interface between the polymer solution and the water droplets. Moreover, DOPE is dissolved in PLA/chloroform solution easily owing to its structure of unsaturated alkyl chains. These two points, having unsaturated alkyl chains and a small hydrophilic area, and high solubility, are suitable for fabricating the honeycomb-patterned film. In this study, DOPE as a surfactant suits the PLA/chloroform solution for fabricating honeycomb-patterned film. Phospholipids are one of the major building blocks of the cell wall, and are safe and biodegradable. In addition, they are used for the drug delivery system, so this honeycomb-patterned film composed of poly(lactic acid) and DOPE is also safe and completely biodegradable.

We have examined the suitable concentration of DOPE for the honeycomb formation. Table 2 shows the relationship between the capability of the honeycomb-patterned structure and the DOPE concentration. When the

Table 2
The relationship between the capability to form the honeycomb-patterned structure and the concentration of DOPE

DOPE concentration[a] (wt%)	Honeycomb pattern	Pore diameter[b,c] (μm)
0.1	No uniform pattern	1–13
0.2	Honeycomb pattern	5.5±0.5
0.5	Honeycomb pattern	4.5±0.5
1	Honeycomb pattern	5.5±0.5
2	Honeycomb pattern	7±1
5	Honeycomb pattern	8±2
10	Honeycomb pattern	7±1
20	Honeycomb pattern	8±2
100	Not observed	—

[a]The concentration of DOPE by weight based on the weight of PLA.
[b]Pore diameters were estimated by optical microscope.
[c]Data were expressed as mean and standard deviations (SD), $n = 3$, except for the data of 0.1 wt% DOPE.

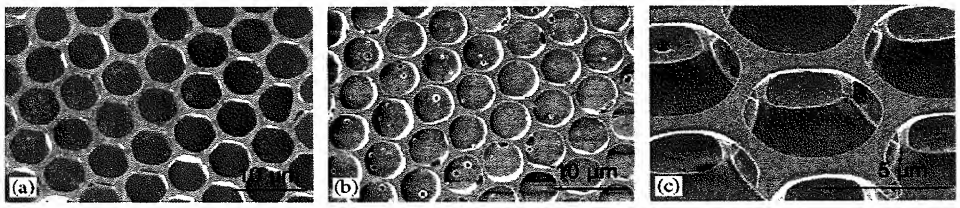


Fig. 1. FE-SEM images of the honeycomb-patterned films: (a) an image of the honeycomb-patterned film using DOPE as the surfactant (PLA–10%DOPE), bar = 10 μm, (b) an image of PLA–0.5%DOPE, bar = 10 μm and (c) a higher-magnification image of PLA–0.5%DOPE tilted at 40°, bar = 5 μm.

concentration of DOPE was from 0.2% to 20% by weight based on the weight of PLA, a uniform honeycomb-patterned structure was observed. The diameter of the honeycomb pore was from 6 to 10 μm when the concentration of DOPE was from 2 to 20 wt%. On the other hand, the diameter was from 4 to 6 μm when the concentration of DOPE was from 0.2 to 1 wt%. When the DOPE concentration was 0.1 wt%, no uniform pattern was observed, and the pore sizes varied from 1 to 13 μm (Table 2). On the contrary, when it was higher than 20 wt%, the honeycomb-pattern was not observed at all. It has been reported that more than 5 wt% of surfactant concentration was needed to fabricate the honeycomb-patterned structure of PLA when CAP was used [15]. By using DOPE, however, a lower amount and concentration can form the same structure on PLA film.

An FE-SEM image of the honeycomb structure of PLA–10%DOPE is shown in Fig. 1(a) and FE-SEM images of PLA–0.5%DOPE are shown in Fig. 1(b) and (c). Fig. 1(c) is the image of PLA–0.5%DOPE tilted at 40°. Uniform honeycomb structures are clearly observed as shown in Fig. 1(a) and (b). The pore size is around 5 μm, and the thickness of the honeycomb skeleton is about 1 μm. All pores are interconnected with each other (Fig. 1(c)).

The contact angles of water on the surface of the honeycomb-patterned PLA films are shown in Fig. 2. The water contact angles of non-washed PLA–DOPE were smaller than those of PLA–CAP, therefore, the surfaces of PLA–DOPE were supposed to be more hydrophilic than PLA–CAP. Additionally, the water contact angle decreased with an increase in the DOPE concentration. These findings suggest that DOPE may locate on the surface of the film. In order to prove this hypothesis, the surfaces of the honeycomb-patterned films were washed with ethanol, in which DOPE can be dissolved. This treatment dramatically increased the contact angle and decreased the hydrophilicity. The contact angles of PLA–DOPE after ethanol washing were as high as that of PLA–CAP at any concentration of DOPE.

NMR data supported the fact that ethanol washing reduced the content of DOPE. The content of DOPE was reduced to 30% in PLA–0.5%DOPE, and to 70% in PLA–2%DOPE. As each film contains 0.125 and 0.5 mg of DOPE, the residual DOPE is estimated to be 0.0375 and 0.35 mg, respectively. On the other hand, the content of

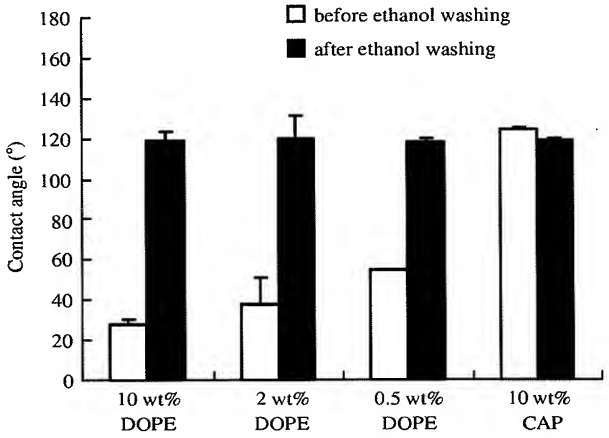


Fig. 2. Changes in water contact angles of various honeycomb-patterned films before ethanol washing (open columns) and after washing (closed columns). Data were expressed as mean and standard deviations (SD), $n = 3$.

CAP was hardly reduced in PLA–CAP after ethanol washing, and 95% of CAP (23.8 mg) remained.

A TOF-SIMS image also yielded evidence that DOPE exists on the surface of the honeycomb-patterned film. A TOF-SIMS image of DOPE fragments is shown in Fig. 3(a), PLA fragments are shown in Fig. 3(b), and the total ion fragments (merged image) are shown in Fig. 3(c). These results suggest that DOPE is condensed on the surface of the honeycomb-patterned film, and this phenomenon imparts a different hydrophilicity to each film depending on the content of DOPE. Therefore, the hydrophilicity of the honeycomb-patterned film can be controlled by the content of DOPE.

To examine the safety of the honeycomb-patterned PLA film, NIH3T3 cells were cultured on the film. Two films with different DOPE content (PLA–0.5%DOPE and PLA–2%DOPE) and PLA–10%CAP were examined. Ethanol washing was performed to sterilize the films before the cells were cultured. After 14 days of cell culture, NIH3T3 cells adhered to and covered the surface of the honeycomb-patterned film (PLA–2%DOPE), as shown in Fig. 4(a). On PLA–0.5%DOPE (Fig. 4(b)), some cells migrated through the pores of the honeycomb structure and adhered to the bottom surface of the

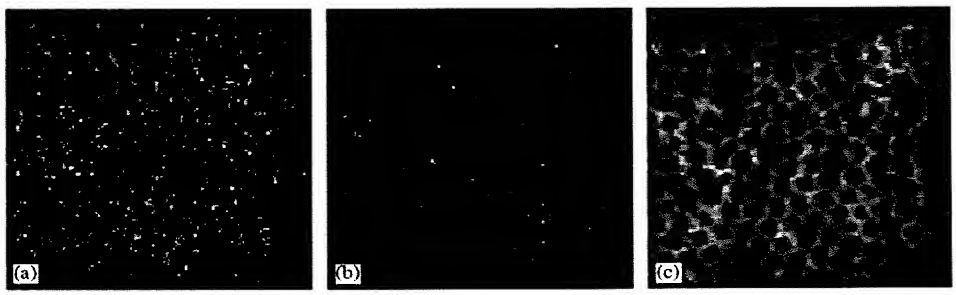


Fig. 3. TOF-SIMS images of the PLA–2%DOPE film: (a) DOPE ion image, (b) PLA ion image and (c) total ion image (merged image).

Fig. 4. (a) SEM image of NIH3T3 cells on the PLA–2%DOPE, bar = 500 μm, (b) FE-SEM image of the interaction between cells and the honeycomb-patterned structure (PLA–0.5%DOPE), and (c) FE-SEM image of the interaction between cells and the honeycomb-patterned structure (PLA–10%CAP) after 14 days of culture, bar = 10 μm.

honeycomb-patterned layer and fiber-like extracellular matrices, around 50 nm in diameter, expanded from the NIH3T3 cells. On the contrary, they did not spread from the cells on PLA–10%CAP (Fig. 4(c)). The area of the film covered with NIH3T3 cells was calculated using image analysis software. Sixty-six percent of the surface area of PLA–2%DOPE was covered with fibroblasts, 53% in PLA–0.5%DOPE and 28% in PLA–10%CAP. These data suggest that PLA–DOPE films are covered with cells via adhesion, and migration processes are comparatively faster than PLA–CAP film.

Moreover, cell proliferation was evaluated by alamarBlue™ assay and summarized in Fig. 5. After 24 h of cell culture, cell proliferation on PLA–0.5%DOPE was comparatively higher than that on PLA–2%DOPE and PLA–10%CAP ($p<0.05$). After 48 h, cell proliferation on PLA–0.5%DOPE was significantly higher than that on PLA–2%DOPE ($p<0.01$). It was clearly observed that cell proliferation on PLA–DOPE films was higher than PLA–CAP ($p<0.001$).

The cell proliferation assay revealed that PLA–DOPE films were better than PLA–CAP for NIH3T3 cell culture. DOPE is one of the major building blocks of the cell wall and is safe, as is clear from being used for the drug delivery system. On the other hand, CAP, a polymer derived from dodecylacrylamide and ω-carboxyhexylacrylamide, is broken down into acrylamide and other moieties. As the biocompatibility of acrylamide is still unclear, this difference might lead to better cell proliferation and adhesion on PLA–DOPE than on PLA–CAP.

It is well known that the pore size of artificial substrates has considerable influence on the cell behavior. For

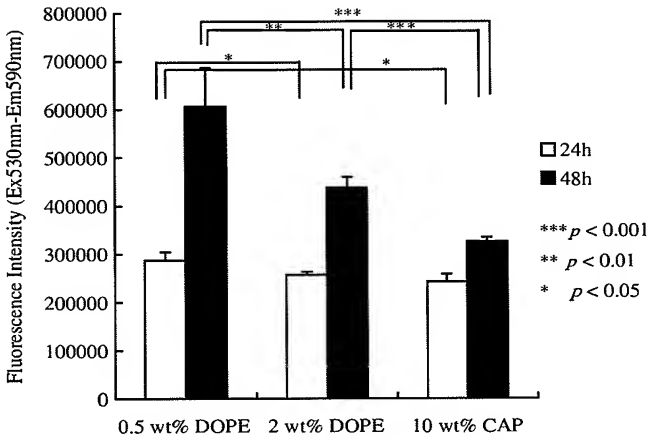


Fig. 5. Cell proliferation of NIH3T3 fibroblast cells cultured on honeycomb-patterned film assessed by alamarBlue™ assay. Data of 24 h (open columns) and 48 h (closed columns) cell cultures after they were expressed as mean and standard deviations (SD), $n = 3$ or 4.

example, the degree of spreading of hepatocyte was enhanced with the decrease of the pore size [18,19]. Moreover, the morphologies of neural cells could be changed by varying the pore size of the patterned films [20]. In this study, we used only 3-μm pore-size films for the cell culture, therefore further studies are required to confirm the relationship between cell behavior and the dimensions of the honeycomb structure in the case of DOPE as a surfactant.

Porous biodegradable polymer materials have been fabricated by various methods, such as phase separation,

salt leaching and freeze drying; however, the pore size is difficult to control by these methods. Our method is simple and can fabricate uniform pores. Moreover, our film is very thin and can be applied to any place. These characteristics can be advantages for application to tissue engineering. In other series of studies, we confirmed that the fine-structured pattern fabricated on the substrate had been kept for 3 weeks in a chondrocyte culture (data not shown).

4. Conclusions

In the present work, we found that DOPE is suitable as a new surfactant for fabricating micro-size honeycomb-patterned film among a series of phosphatidylcholines and phosphatidylethanolamines. The honeycomb-patterned PLA films were fabricated with concentrations of DOPE of from 0.2% to 20% by weight based on the weight of PLA. Contact-angle measurement and TOF-SIMS observation suggested that DOPE was concentrated and located on the surface of the honeycomb-patterned film. The content of DOPE can control the hydrophilicity of the film. Cell proliferation and adhesion on PLA–DOPE film were higher than on PLA–CAP. PLA–DOPE was demonstrated to be a good scaffold for cells such as NIH3T3.

Acknowledgements

We gratefully acknowledge our thanks to Masaya Ito for his kind help and advice, plus Hisao Moriya, Masahito Sato and Makiko Ikeda of the Institute for Structure Analysis, TEIJIN Limited, for the SEM, FE-SEM and TOF-SIMS observations, image analysis and helpful discussions.

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Interfacial tension governs the formation of self-organized honeycomb-patterned polymer films

Yukako Fukuhira,*[ab] Hiroshi Yabu,[c] Kuniharu Ijiro[d] and Masatsugu Shimomura[ce]

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Hexagonally packed water droplets condensed on a polymer solution are potential templates for the formation of honeycomb-patterned porous polymer films. A small number of surface-active molecules is indispensable for the stabilization of water droplets during solvent evaporation. Biocompatible surfactants; *e.g.*, phospholipids, are required for the fabrication of biodegradable honeycomb-patterned polymer films, which can be used as novel biomedical materials, mainly *in vivo*. Among various kinds of phospholipids, dioleoylphosphatidylethanolamine (DOPE) has been reported to be the most suitable surfactant for the formation of honeycomb-patterned PLA films. Interfacial tension between a water droplet and the polymer solution is largely dependent on the chemical structure of the phospholipids. DOPE shows high interfacial tension, resulting in the stabilization of water droplets during solvent evaporation. Dierucoylphosphatidylcholine (DEPC) and dierucoylphosphatidylethanolamine (DEPE), both of which display high interfacial tension, were also found to be suitable biocompatible surfactants.

Introduction

A honeycomb-patterned polymer film is fabricated by casting a polymer solution the surface of which is sprayed with high humidity air. Regularly arrayed micropores are formed in the polymer film by using a self-organized array of water droplets as a template.[1–7] To form a uniform array of water droplets, it is important that they be stabilized on the polymer solution surface during solvent evaporation. By evaporation cooling, water molecules condense to form water droplets of uniform size on the polymer solution surface. In order to prevent the coalescence of the water droplets and to stabilize them, a surfactant is added to the polymer solution. The surfactant molecules are localized at the interface between the water droplets and the polymer solution. After solvent evaporation, the regular arrangement of the water droplets is maintained and, after water evaporation, the honeycomb-patterned structure is formed. The stability and arrangement of the water droplets are strongly affected by the concentration and structure of the surfactants.

We have already reported that amphiphilic polymers, copolymers of dodecylacrylamide and ω-carboxyhexylacrylamide (CAP), as well as poly-ion complexes, are effective surfactants for the formation of honeycomb-patterned films.[8,9] These films have potential applications as medical devices such as cell culture substrates,[10–15] cell separators,[9] temporary epicardial pacing wires,[16] adhesion barriers,[17,18] and superhydrophobic films.[19] However, biocompatible polymers and surfactants are required for the *in vivo* use of honeycomb-patterned films.

[a]*N10W8, Sapporo, 060-0810, Japan*
[b]*4-3-2, Asashigaoka, Hino, 191-8512, Japan. E-mail: y.fukuhira@teijin.co.jp; Fax: +81 42587 5511; Tel: +81 42 586 8325*
[c]*2-1-1, Katahira, Aobaku, Sendai, 980-8577, Japan*
[d]*N21W10, Sapporo, 001-0021, Japan*
[e]*2-1-1, Katahira, Aobaku, Sendai, 980-8577, Japan*

Recently, we have focused on the use of phospholipids as biocompatible surfactants for the formation of biodegradable honeycomb-patterned films. Among the various kinds of phospholipids, we have found that dioleoylphosphatidylethanolamine (DOPE) is the most effective as a biocompatible surfactant.[20]

In order to elucidate the effectiveness of DOPE in the formation of honeycomb-patterned films, the interfacial tension between the water droplets and polymer solution was measured, because interfacial tension is one of the key physical parameters for determining the stability and arrangement of the water droplets, and the results are presented herein.

Experimental section

Poly(D,L-lactic acid) (PLA) (MW = 200,000, Lacty #9031, Shimadzu, Kyoto, Japan) was dissolved in chloroform (Wako Pure Chemical Industries Ltd., Osaka, Japan) at a concentration of 5 mg/mL at room temperature. Phospholipids, DOPE (COATSOME ME-8181), dilauroylphosphatidylcholine (DLPC; COATSOME MC-2020), dimyristoylphosphatidylcholine (DMPC; COATSOME MC-4040), dipalmitoylphosphatidylcholine (DPPC; COATSOME MC-6060), distearoylphosphatidylcholine (DSPC; COATSOME MC-8080), dioleoylphosphatidylcholine (DOPC; COATSOME MC-8181), dierucoylphosphatidylcholine (DEPC; COATSOME MC-2121AL) and dierucoylphosphatidylethanolamine (DEPE; COATSOME ME-2121AL), were purchased from NOF Co. (Tokyo, Japan) and separately added to a polymer solution (0.5 wt% PLA). The honeycomb-patterned films were prepared on a 100 mm petri dish at room temperature under humid air (approximately 80% humidity). The structures of the films were observed using an optical microscope and a field-emission scanning electron microscope (FE-SEM). In this experiment, the

shape of a water-soluble blue dye (Indigocarmine; Wako Pure Chemical Industries Ltd., Japan)-stained, millimeter-scale water droplet located on the surface of the polymer solution containing the phospholipid surfactants was observed.

The interfacial tension of each polymer solution was measured using the pendant drop technique[21] (FAMAS CA-W software, Kyowa Interface Co., Ltd., Japan). A drop of water was obtained from a clean 22-gauge Teflon cannula immersed in the polymer solution in a 10 mL quartz glass cuvette at 20 °C. The interfacial tension (γ) was determined as follows,

$$\gamma = \Delta\rho g d_e^2/H \quad (1)$$

where $\Delta\rho$ is the mass density difference between the drop and the fluid surrounding the drop, g is the gravitational acceleration, d_e is the equatorial diameter of the drop, and H is a dimensionless shape factor. H is a function of d_s/d_e, where d_s is the diameter of the pendant drop measured at a vertical distance d_e from the apex of the drop. The lengths corresponding to d_e and d_s of the drop were measured from digital images. The average and standard deviations of each γ value were determined by using experimental data from ten sets of measurements.

Results and discussion

In this study, DOPE and a series of phosphatidylcholines were used as surfactants, because no other phospholipids could be dissolved in the polymer solution.[20]

Fig. 1 shows FE-SEM images of the surface of the fabricated PLA films using each phospholipid as a surfactant. Schematic views of the cross-section of the film are also shown. As reported previously,[20] a uniform honeycomb-patterned structure was formed over the entire area of the DOPE/PLA film (Fig. 1(a)), whereas uniform and nonuniform honeycomb-patterned structures coexisted in the DOPC/PLA film (Fig. 1(b)). Nonuniformly sized pores were irregularly arranged in the DSPC/PLA film (Fig. 1(c)), and small, regularly sized pores were collected in sections of the surface of the DPPC/PLA film (Fig. 1(d)). On the DMPC/PLA film surface, a random network structure of the order of several micrometers was observed (Fig. 1(e)), and randomly located 2 μm concave grooves were seen on the surface of the DLPC/PLA film (Fig. 1(f)). The honeycomb-patterned structure was only successfully formed on the PLA film surface when DOPE was used as the surfactant.

Since the honeycomb-patterned film was fabricated using a self-organized array of water droplets as a template, the shape of the water droplets would influence the structure of the pores in the film. The effect of phospholipids on water droplet shape was estimated by observation of a millimeter-scale water droplet in the polymer solutions containing each phospholipid, because direct observation of the shape of micrometer-scale water droplets is difficult (Fig. 2). Two types of polymer solutions, a low concentration solution (5 mg/mL) and a high concentration solution (100 mg/mL), were prepared, as the polymer solution was concentrated during the honeycomb-patterned film fabrication process. Side views of the water droplet in the low concentration solution are shown in Fig. 2(I) for each phospholipid. The shape of the water droplet in the low concentration solution was almost globular. In the high concentration DOPE/PLA solution, the globular shape of the water droplet was maintained (Fig. 2(a)-(II)). In contrast, in the high concentration DOPC/PLA solution, the droplet expanded slightly and its shape changed (Fig. 2(b)-(II)). The expansion of the water droplet in the high concentration DSPC/PLA solution was significantly increased (Fig. 2(c)-(II)), and this trend was observed in the high concentration solutions containing DPPC, DMPC and DLPC (Fig. 2(d)-(II), 2(e)-(II) and 2(f)-(II)). Fig. 2 clearly shows that

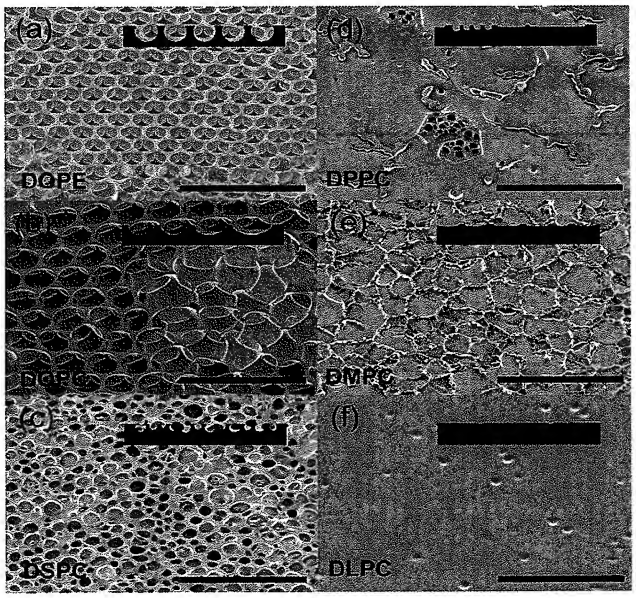

Fig. 1 FE-SEM images of film surfaces. (a) PLA/DOPE, (b) PLA/DOPC, (c) PLA/DSPC, (d) PLA/DPPC, (e) PLA/DMPC, and (f) PLA/DLPC. The scale bar represents 20 μm. Schematic views show the cross-sectional structure of each film.

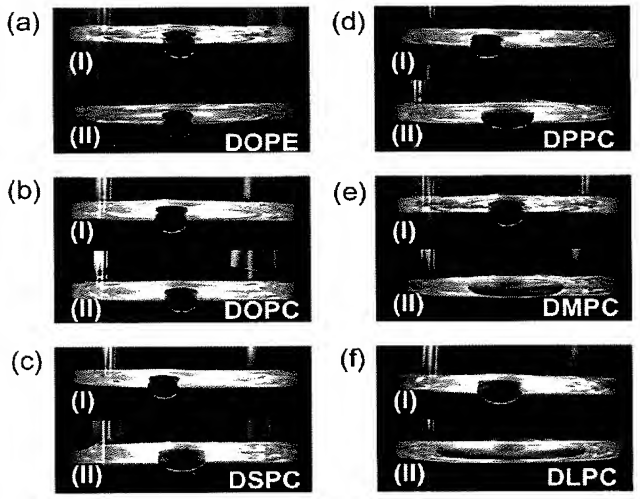

Fig. 2 Appearance of water droplet in the polymer solution. (a) DOPE/PLA, (b) DOPC/PLA, (c) DSPC/PLA, (d) DPPC/PLA, (e) DMPC/PLA, and (f) DLPC/PLA. (I) Phospholipid/PLA/chloroform = 0.025 mg/5 mg/ml. (II) Phospholipid/PLA/chloroform = 0.5 mg/100 mg/ml.

Table 1 HLB data of phospholipids

Surfactant	RCOO	HLB
DOPE	C18:1	6.5
DOPC	C18:1	7.2
DSPC	C18:0	7.2
DPPC	C16:0	7.7
DMPC	C14:0	8.3
DLPC	C12:0	9.1

when the alkyl chain length of the phospholipid became shorter, the water droplet spread in the high concentration solution. These results indicate that phospholipids strongly influence the shape and stability of water droplets in the polymer solution.

The ability of the phospholipid to dissolve the water droplets in the polymer solution can be predicted from the hydrophile–lipophile balance (HLB). The HLB values of the phospholipids were calculated using Griffin's method (Table 1).

In general, surfactants with HLB values between 3 and 6 partially disperse in water and are used as water-in-oil (W/O) emulsifiers. Surfactants with HLB values between 7 and 9 disperse well in water and are used as wetting agents and oil-in-water (O/W) emulsifiers. Surfactants with such high HLB values are not suitable for fabricating honeycomb-patterned films, because these surfactants are dissolved into the water droplets and the surface tension of the water droplets is decreased. Harkins expresses the spreading coefficient (S) as follows,

$$S = \gamma_a - (\gamma_b + \gamma_{a/b}) \quad (2)$$

where γ_a is the surface tension of the polymer solution, γ_b is the surface tension of the water droplet, and $\gamma_{a/b}$ is interfacial tension between the polymer solution and the water droplets in this case. This means that a water droplet with low surface tension cannot maintain a globular shape. Therefore, DOPE, which has a low HLB value (6.5), can maintain water in the form of droplets in a polymer solution.

It is essential to investigate the relation between the chemical structure of the phospholipid and interfacial tension in order to quantitatively discuss the estimated size of the millimeter-scale water droplets in the polymer solution containing the phospholipid, because the stability of the water droplets in the polymer solution is governed by interfacial tension. In the film fabrication process, the concentrations of the polymer and surfactant in the solution were gradually increased with solvent evaporation. Therefore, the interfacial tension was measured at four simulated stages of evaporation (0.025 mg/5 mg/mL (phospholipid/PLA/chloroform), 0.05 mg/10 mg/mL, 0.25 mg/50 mg/mL, and 0.5 mg/100 mg/mL); *i.e.*, from the initial concentration state to a highly concentrated state (Fig. 3). The interfacial tension decreased with increasing phospholipid concentration for each phospholipid. Among the phospholipid-containing solutions used in our study, the DOPE/PLA solution showed the highest interfacial tension, as DOPE is the most hydrophobic of the phospholipids owing to its having the longest unsaturated alkyl chains and a small head group.

To investigate the relation between interfacial tension and honeycomb-patterned structure formation, *in situ* observation of the honeycomb-patterned film fabrication process was carried out for DOPE/PLA and DMPC/PLA films. The time course of

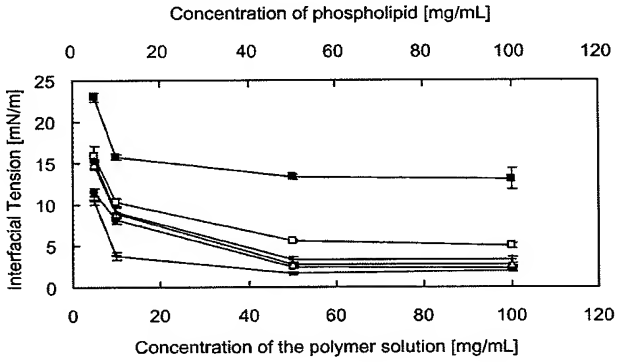

Fig. 3 Interfacial tension of phospholipids at the polymer solution–water interface as a function of total polymer concentration. ■: DOPE, □: DOPC, +: DSPC, △: DPPC, ●: DMPC, —: DLPC.

the interfacial tensions of the DOPE/PLA films, the polymer solution concentration, and the film surface appearance are shown in Fig. 4(a). The polymer solution concentration was calculated from the polymer solution weight, which was monitored *versus* time. Optical interference due to the regularly formed array of the water droplets was observed on the surface of the polymer solution approximately 60 s after the start of the film fabrication process (Fig. 4(a)-(I)). The interference spread to the entire area of the petri dish by approximately 120 s (Fig. 4(a)-(II)). The solidification of the film surface occurred at approximately 180 seconds (Fig. 4(a)-(III)). At this point, the concentration of the polymer solution was approximately 10 mg/mL. Interference due to the honeycomb-patterned structure was observed after the evaporation of chloroform and water droplets from the film surface (Fig. 4(a)-(IV)). The value of the interfacial tension was maintained above 10 mN/m after film solidification. On the other hand, in the case of the DMPC/PLA films, interference was observed only in the initial stage (Fig. 4(b)-(I)) and disappeared with time (Fig. 4(b)-(II)). This variation in interference time course between the two phospholipids can be explained by the fact that as the interfacial tension at the initial concentration of the polymer solution was higher than 10 mN/m, water droplets remained stable at the polymer solution surface. During solvent evaporation, the interfacial tension decreased below 10 mN/m, causing the water droplets to be deformed as they coalesced on the polymer solution. In all polymer solutions containing phospholipids, with the exception of that containing DOPE, the interference appeared only once and then disappeared. When the interfacial tension was higher than 10 mN/m, at the time at which solidification of the polymer solution occurred, the water droplets were sufficiently stable to cause stable interference, thereby yielding good-quality honeycomb-patterned films.

The stability of a free, thin liquid film against small, spontaneous fluctuations in thickness has been previously explored.[22] The liquid film is unstable with respect to fluctuations with a wavelength larger than the critical wavelength. The condition under which the film coalesces is described by the Vrij theory as follows,

$$d^2V/dh^2 < -2\pi\gamma/A_c \quad (3)$$

where $V(h)$ is the free energy of the interaction as a function of the film thickness h, γ is the interfacial tension, and A_c is the

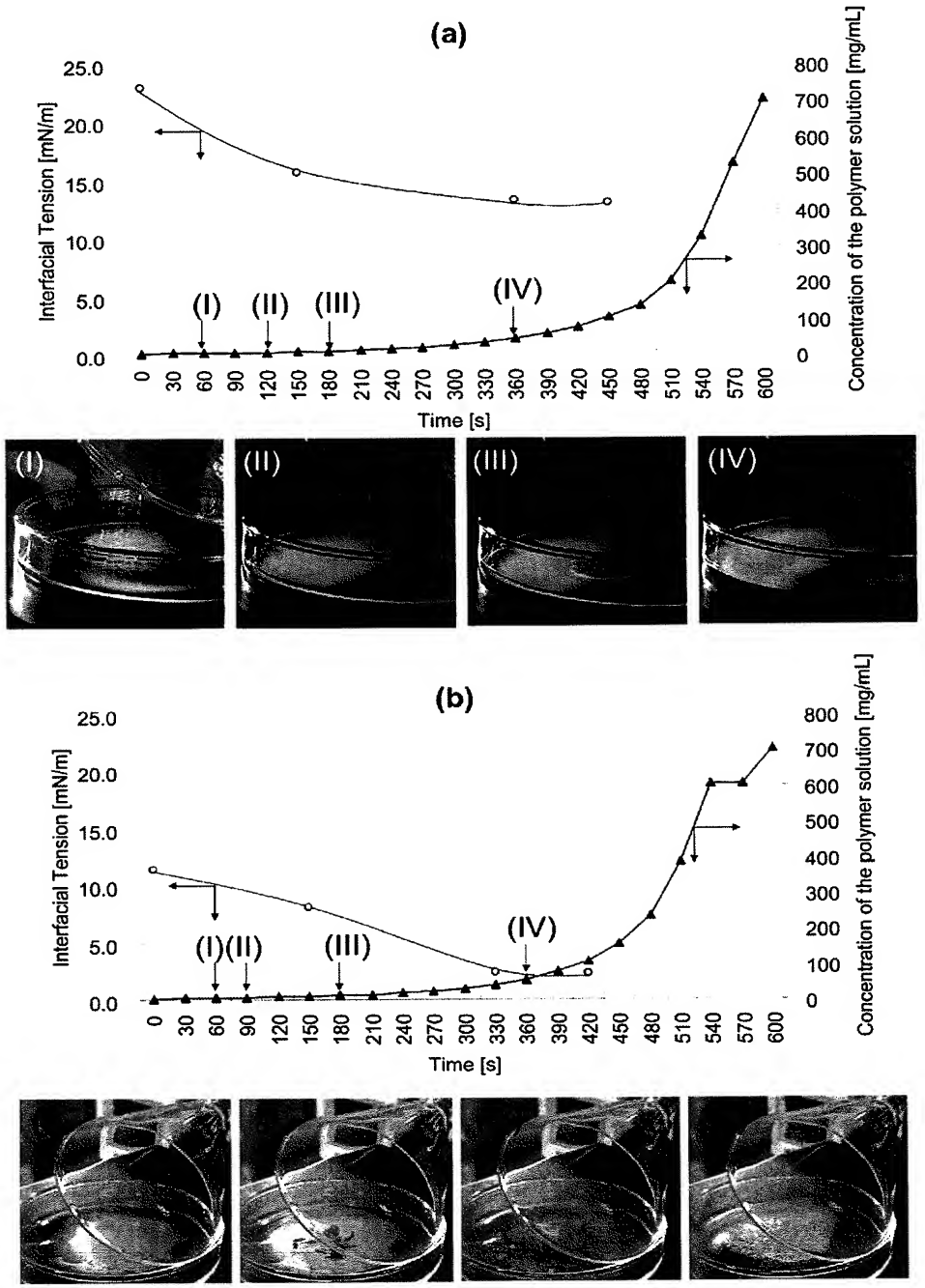

Fig. 4 (a) Change in interfacial tension of DOPE (○) at the polymer solution–water interface and the concentration of the polymer solution (▲) as a function of film formation time. Video frames captured at 60, 120, 180, and 360 s after the start of the experiment are also shown. (b) Change in interfacial tension of DMPC (○) at the polymer solution–water interface and the concentration of the polymer solution (▲) as a function of film formation time. Video frames captured at 60, 90, 120, 180, and 360 s after the start of the experiment are also shown.

wavelength of the critical fluctuation. $V(h)$ includes van der Waals attraction and double-layer repulsion. Equation (3) explains why a low interfacial tension has a tendency to break the liquid film.[23] In our study, surfactants having a low interfacial tension could not maintain the coalesced water droplets, thus our results are in excellent agreement with the results predicted using equation (3).

To confirm this, we next investigated surfactants with interfacial tension higher than 10 mN/m in a 10 mg/mL polymer solution. The interfacial tension of DEPC containing

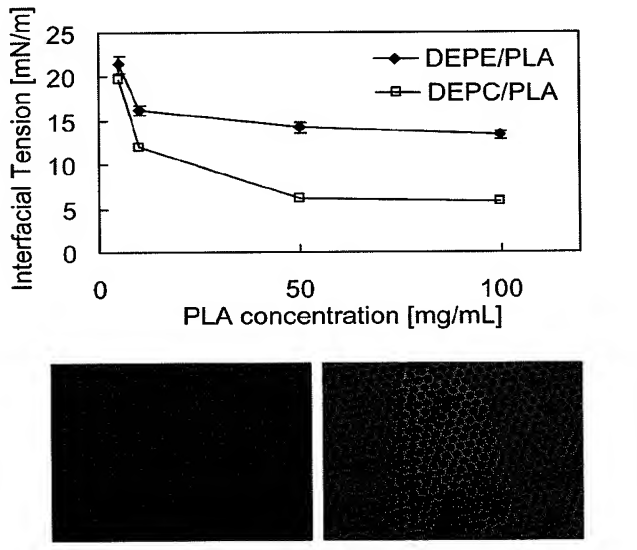

Fig. 5 Interfacial tension of DEPC and DEPE at the polymer solution–water interface as a function of total polymer concentration. Optical microscope images of film surfaces. (a) PLA/DEPE, and (b) PLA/DEPC.

unsaturated alkyl chains having 22 carbon atoms and 1 double bond was 12 mN/m in 10 mg/mL of the PLA polymer solution (0.5 wt% of PLA), and the interfacial tension of DEPE containing unsaturated alkyl chains having 22 carbon atoms and 1 double bond was 16 mN/m in 10 mg/mL of the PLA polymer solution (0.5 wt% of PLA). The HLB values of DEPC and DEPE were 6.3 and 5.6, respectively, which compare favorably with that of DOPE (6.5). Thus both the DEPC/PLA and DEPE/PLA solutions were predictably effective in the fabrication of the honeycomb-patterned films (Fig. 5).

Conclusion

The stabilization of the water droplets on a polymer solution surface was necessary for the formation of a honeycomb-patterned structure. The stability of the water droplets in the solution is affected to a large degree by the surfactants. The HLB value and interfacial tension are important parameters affecting droplet stability. DOPE, which is effective as a surfactant for honeycomb-patterned film formation, has a low HLB value and can maintain high interfacial tension (>10 mN/m) during chloroform evaporation. Therefore, the results of this study have aided us in formulating guidelines for selecting surfactants to be used in the fabrication of honeycomb-patterned films.

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